JOURNAL OF VIROLOGY, Nov. 1982, p. 725–730
0022-538X/82/110725-06$02.00/0

Vol. 44, No. 2

Avian Sarcoma Virus *gag* Precursor Polypeptide Is Not Processed in Mammalian Cells

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Received 21 April 1982/Accepted 8 July 1982

We studied intracellular avian *gag* proteins (internal structural proteins of virions) in several mammalian cell lines transformed by Rous sarcoma virus. All lines examined contain *gag* antigens as determined by radioimmune assay. We used the techniques of protein blotting from polyacrylamide gels, which detects nanogram quantities of viral protein, to investigate the size of intracellular viral polypeptides. All of the lines that contained enough viral protein to be amenable to this type of analysis synthesized Pr76, the avian sarcoma virus *gag* precursor polypeptide, but failed to process it into mature virion proteins. In some cell lines, the recovery of Pr76 was greatly enhanced by the addition of a mixture of protease inhibitors, including the sulfhydryl-blocking reagent *N*-ethylmaleimide, to the lysis buffer. At least several of the mammalian cells also synthesized a viral polypeptide the size of Pr180, the precursor to reverse transcriptase. Since Rous sarcoma virus does not replicate or replicates extremely poorly in mammalian cells, the lack of processing suggests that cleavage and virion assembly are invariably associated.

Rous sarcoma virus ([RSV] or nondefective avian sarcoma virus) is able to induce the neoplastic transformation of cells of diverse species, including birds, mammals, and reptiles. In contrast, by biochemical, electron microscopic, and biological analysis, RSV is able to replicate, forming mature virions, only in avian cells (2, 6, 7, 14). Rare RSV-transformed mammalian cell lines have been reported to produce transforming virus particles but at a titer several orders of magnitude lower than infected chick cells (13). The identity of these viruses is uncertain. The low level of viral protein in RSV-transformed mammalian cells (see below) is not a sufficient explanation for the lack of assembly of virions since certain chick cells with low levels of protein produce infectious particles normally. Of the three genes common to all nondefective avian sarcoma and leukemia viruses (*gag*, internal structural proteins; *pol*, reverse transcriptase; and *env*, envelope proteins), only the *gag* gene must be functional in infected chick cells to allow the assembly and budding of virions at the plasma membrane (reviewed in reference 4). This inference stems from the fact that in cells infected with mutant viruses with deletions of *pol* or of *env* genes, virus particles are formed that are defective, but whose internal structure appears normal by electron microscopy. Thus, it seems likely that the block in virus assembly in mammalian cells involves the *gag* gene product.

The four major internal proteins of RSV are derived by proteolytic cleavage of Pr76, the *gag* precursor polypeptide that is the primary translational product of the *gag* gene (16). It was shown originally (5) that one RSV-transformed cell line of hamster origin, H-RSV(BH), synthesizes but does not process Pr76. This study employed the labeling of cells with [^{35}S]methionine followed by the immune precipitation of cell lysates with anti-*gag* serum and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Several other RSV-transformed mammalian lines labeled in parallel failed to reveal any *gag* polypeptides by this analysis, presumably because they are present in too low a concentration to be distinguishable from the nonspecific background of polypeptides in the immune precipitate. The stability of Pr76 in H-RSV(BH) cells suggested a model in which the inability of mammalian cells to assemble avian sarcoma virus particles is related to their inability to process Pr76 correctly.

We have now reinvestigated the production of *gag* proteins in diverse mammalian cells, using radioimmune techniques that are more sensitive than metabolic labeling followed by immune precipitation. Table 1 lists some of the cell lines examined and the quantities of *gag* protein (as p27 or p15 antigenic determinants) measured by standard radioimmune assays. These quantities must be regarded as approximate, since the assays were done in the absence of sulfhydryl blocking reagents (see below) and since the

TABLE 1. Radioimmune assay of avian *gag* antigen[a]

Species	Cell line[b]	ng of p27/mg of cell protein	ng of p15/mg of cell protein
Hamster	H-RSV(BH)	27	28
Rat	B77-NRK	12	8
	XC	1.1	ND[c]
Mouse	SR-BALB	9.6	5
	SR-C57	4.5	ND
	SR-3T3	1.2	ND
	B77-3T3	1.4	ND
Rhesus	HF-RSV	10	ND
Human	KC	17	4.6
Chick	AMV-CEF	470	ND
Control cells	NRK	<0.2	ND
	SV-3T3	<0.2	ND
	CEF	<0.2	ND

[a] Cells were grown in Dulbecco modified Eagle medium plus 10% fetal calf serum at 35 or 39°C (avian cells). Extracts were prepared from phosphate-buffered-saline-washed cells by lysis with 1 ml of 0.02 M Tris-hydrochloride (pH 7.6)–0.05 M NaCl–0.5% sodium deoxycholate–1 mM PMSF per 10-cm plate at 0°C. Cells and debris were scraped from the plate in this solution, agitated for 30 s, and centrifuged for 10 min at 10,000 × *g* to remove nuclei and debris. The supernatants were stored at −20°C. The radioimmune assays contained 1 to 16 ng of ^{125}I-labeled p27 or p15 (purified by the gel filtration of avian myeloblastosis virus proteins in 6 M guanidine hydrochloride and iodinated with chloramine T in the presence of 0.1% SDS), 5 μl of normal rabbit serum, 10 μl of 0.1% rabbit anti-avian myeloblastosis virus serum, and variable amounts of crude cell extract in 200 μl of 0.02 M Tris-hydrochloride (pH 7.6)–0.1 M NaCl–1 mM EDTA–0.2% Triton X-100–2 mg of bovine serum albumin per ml. After 4 h of incubation at 37°C, 40 μl of goat anti-rabbit immunoglobulin G was added, and the incubation was continued for 14 h at 4°C. The resulting immune precipitate was diluted by the addition of 0.5 ml of the same buffer lacking serum albumin, collected by centrifugation, washed twice in the same buffer, and then counted. At a high concentration, the anti-avian myeloblastosis virus serum precipitated about 70% of the ^{125}I radioactivity. At the dilution used in the competitive assays, the antiserum precipitated 40% of the radioactivity. The values in the table are the averages of duplicate determinations on parallel plates of cells; the two values differed by less than a factor of two.

[b] The cell lines were obtained from the following sources: NRK and SV-3T3, L. Youngman (Cornell University); H-RSV(BH) and B77-NRK, R. Eisenman (Hutchinson Cancer Center); SR-C57, SR-3T3, SR-BALB, XC, and B77-3T3, T. Parsons (University of Virginia); KC, L. Rohrschneider (Hutchinson Cancer Center); HF-RSV, R. Massey (Frederick Cancer Center). AMV-CEF, avian myeloblastosis virus-infected chicken embryo fibroblasts.

[c] ND, Not determined.

competing purified *gag* antigens p27 and p15 are found as larger, and perhaps antigenically different, fragments in cell extracts. All of the cells contained detectable *gag* protein, at levels 20- to 300-fold lower than in RSV-infected chick cells, roughly consistent with other reports of *gag* antigen determinations in mammalian cells (12). The hamster cell line contained the most *gag* protein, as suggested by our earlier findings (5). We sought in preliminary experiments to determine the size of these *gag* polypeptides by the gel filtration of crude extracts in 6 M guanidine hydrochloride followed by radioimmune assay, as described by Reynolds et al. (12). The results suggested that the cleavage of the *gag* precursor Pr76 into the mature virion proteins does not occur (A. P. Bell, Ph.D. thesis, Cornell University, Ithaca, N.Y., 1979). However, the resolution of this technique is not sufficient to allow one to distinguish Pr76 from polypeptide fragments greater than 60,000 daltons in size or from the *gag-pol* precursor, Pr180.

To examine the size of *gag* antigen in non-avian cells more carefully, we employed the technique of protein blotting. In this technique, the polypeptide bands from an SDS-polyacrylamide slab gel are transferred electrophoretically to a nitrocellulose sheet (15). The remaining protein-binding sites are blocked with excess serum albumin, the nitrocellulose is incubated with a dilute solution of antiserum, and then after the incubation of the nitrocellulose with ^{125}I-labeled staphylococcal A protein (11), the bands where antibodies have reacted with an antigen are visualized by fluorography. Besides its high sensitivity, this technique has the advantage that freshly prepared crude cell lysates can be dissolved immediately in SDS and then electrophoresed, thus minimizing the chances of artifactual proteolysis. In most of the experiments, we used a serum from a rabbit injected with avian myeloblastosis virus, known to contain antibodies to all four major *gag* proteins. With this serum and with others, it appears that p27 was by far the most readily detected viral protein. Proteins p15 and p19 were less intensely and less reproducibly visualized. We did not attempt to interpret the blotting analysis quantitatively, since it is uncertain whether the same antigenic determinants in p27 and in the larger p27-related polypeptides will bind antibody and ^{125}I-labeled staphylococcal A protein equally efficiently.

Initial experiments with protein blotting indicated that the mature *gag* proteins p27 and p15 are completely absent from all mammalian cells and that cells not transformed by avian sarcoma viruses contain no polypeptides that cross-react with the *gag* antiserum used in this analysis. But several RSV-transformed lines, in particular

those derived from rat kidney (NRK), frequently showed, in addition to Pr76, a spectrum of antigenically related polypeptide bands smaller than Pr76. The observation that the same polypeptide bands, presumably derived from Pr76, were visualized by the antiserum even when cells were lysed in the presence of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) initially suggested that these fragments might be generated in vivo. However, this conclusion was erroneous, since the inclusion of the sulfhydryl-blocking reagent *N*-ethylmaleimide (NEM) prevented the appearance of the fragments and led to a large increase in the recovery of Pr76 itself.

The effect of several protease inhibitors on the pattern of avian *gag* proteins in H-RSV(BH) cells (RSV-transformed hamster) and on B77-NRK cells (RSV-transformed rat) is shown in Fig. 1. In this experiment, extracts of cells were prepared in standard detergent-containing lysis buffer containing no addition, EDTA, PMSF, aprotinin, NEM, or mixtures of these compounds. The lysates were immediately adjusted to 5% SDS plus 2% 2-mercaptoethanol, heated at 90°C for 2 min, and then frozen for later gel analysis. The hamster cell extracts (Fig. 1, lanes a through d) all show a predominant band of Pr76, with little indication of proteolysis with or without inhibitors. In the rat cells, by contrast, little Pr76 and several lower-molecular-weight polypeptides are visible in extracts prepared in lysis buffer alone (Fig. 1, lane f). The addition of a mixture of protease inhibitors to the buffer before lysis completely suppressed the lower-molecular-weight bands and led to at least a 10-fold enhancement in the recovery of Pr76 (Fig. 1, lane g). Although the inhibitor mixture in this experiment included PMSF, aprotinin, EDTA, and NEM, we have found in other experiments with B77-NRK cells, as well as with a different strain of RSV-transformed NRK cells, that the stabilization of Pr76 can be effected by NEM alone.

We conclude from these results that after the lysis of some mammalian cells, the RSV *gag* precursor Pr76 can be rapidly degraded. The extent of degradation varies from experiment to experiment. When it occurred in the rat cells, the degradation did not appear to be time dependent, since the incubation of the crude extracts for 2 h at room temperature did not lead to the further decrease of Pr76 (Fig. 1, lanes b and e). Thus, it seems likely that proteolysis occurs immediately upon cell lysis, although we have not excluded the possibility that it occurs upon the addition of SDS. Although Pr76 usually appeared stable in hamster and other lines when SDS was added to fresh crude extracts, the storage of crude extracts at −20°C also led to a pattern of degradation similar to that observed for transformed NRK cells (Fig. 2). As in the rat cells, this degradation could be prevented by the prior addition of a mixture of protease inhibitors, including NEM. Therefore, previous reports (6, 12) of *gag*-related polypeptides smaller than Pr76 in RSV-transformed mammalian cells can be explained by the fact that the authors failed to use appropriate protease inhibitors in their experiments. The quantitative radioimmune assays in Table 1 were performed in the absence of a sulfhydryl-blocking agent. Hence, it is possible that for H-RSV(BH) and B77-NRK cells, the approximately 10-fold discrepancy between the amount of *gag* antigen measured by the quantitative assay and that estimated from blotting (Fig. 2) also is due to proteolysis.

The autoradiogram in Fig. 1 shows that H-RSV(BH) cells and B77-NRK cells also synthesized a larger *gag*-related polypeptide, which has the same electrophoretic mobility (data not shown) as $Pr180^{gag\text{-}pol}$, the precursor to viral reverse transcriptase (4). This polypeptide was not readily visible in every experiment with these or other cell lines. We attribute this to the variability in the electrophoretic transfer of large polypeptides from polyacrylamide to nitrocellulose or to some other variable in the blotting technique. The synthesis of Pr180 in RSV-transformed rat cells has also been observed by others (9).

The blotting analysis of a series of RSV-transformed cell lines—from mouse, rat, hamster, rhesus monkey, and human species—is shown in Fig. 2. In this experiment, cell extracts were prepared with or without protease inhibitors, and then portions were frozen, lyophilized, and redissolved in 5% SDS sample buffer. Pr76 was visible in each kind of cell. In most of the cells, the addition to the lysis buffer of the protease inhibitor mix, including NEM, increased the yield of the *gag* precursor and suppressed related polypeptides of lower molecular weight. Neither with nor without inhibitors were any mature *gag* antigens detectable. Viper cells transformed by RSV (from D. Boettiger) also lack detectable mature *gag* proteins (data not shown). Figure 2 shows that RSV-transformed quail cells analyzed in parallel did show p27 (the major polypeptide recognized by this antiserum) and other *gag* proteins, as expected, since these avian cells are actively producing virus particles. In this case, the addition of NEM had little effect on the recovery of Pr76, but it did appear to alter the mobility of the several intermediate cleavage fragments of Pr76. This mobility change, the basis for which we have not explored, suggests that the assignment of molecular weights (16) to these intermediate cleavage fragments may be in error.

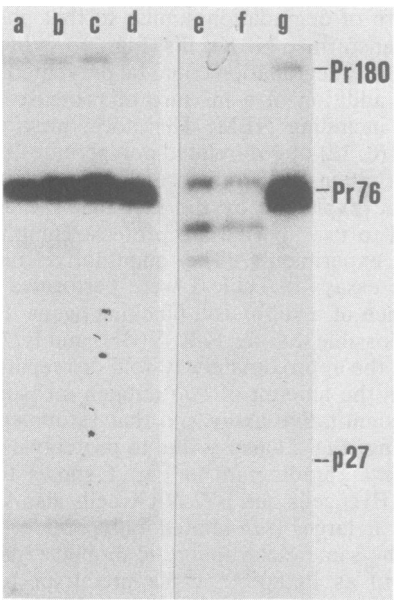

FIG. 1. Effect of protease inhibitors on Pr76. RSV-transformed hamster [H-RSV(BH)] cells and RSV-transformed rat (B77-NRK) cells were grown on 10-cm plates in Dulbecco modified Eagle medium plus 10% fetal calf serum [H-RSV(BH)] or 10% calf serum (B77-NRK). Two plates of each cell line (about 2×10^7 cells or 2 mg of cell protein) were washed with phosphate-buffered saline and then lysed by the addition of 0.25 ml of ice-cold 0.02 M Tris-hydrochloride (pH 7.5)–0.05 M NaCl–0.5% Triton X-100–0.5% sodium deoxycholate per plate. Cells on parallel plates were lysed with the same buffer plus 1 mM PMSF, 20 mM EDTA, 10 mM NEM, aprotinin (Sigma Chemical Co.; 0.2 trypsin inhibitor U/ml), or combinations of these compounds. The cell residue was scraped from the plate, agitated in a Vortex mixer for 30 s, and then centrifuged for 10 min at 10,000 rpm to remove nuclei and debris. Portions of 20 μl were then mixed with 20 μl of 10% SDS–4% 2-mercaptoethanol–25% glycerol–0.12 M Tris-hydrochloride (pH 6.8) and heated for 2 min at 90°C and subsequently stored at −20°C. The samples were thawed later and electrophoresed on a 15% polyacrylamide slab gel in the presence of 0.1% SDS. The gel was soaked for 30 min in 20% methanol–0.025 M Tris base–0.19 M glycine at room temperature, and then the polypeptides were transferred electrophoretically in the same buffer to a nitrocellulose membrane (Schleicher & Schuell BS 85; 5 h at a voltage drop of 20 V/cm, with cooling by ice water). The membrane was rinsed for 15 min in rinse buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100) and agitated for 2 h at room temperature in the same buffer containing 30 mg of bovine serum albumin per ml. It was then sealed in a plastic bag with 4 ml of the same buffer containing bovine serum albumin plus 20 μl of anti-*gag* serum and rotated overnight at 4°C. (In our hands the low temperature of incubation with antiserum reduced the nonspecific background seen on the final fluorograph.) The serum had been obtained from a rabbit multiply injected with avian myeloblastosis virus disrupted with Triton X-100. After incubation with the antiserum, the nitrocellulose membrane was rinsed three times for 30 min at room temperature in a rinse buffer containing 1 M NaCl, and then incubated for 6 h at 4°C in a plastic bag with approximately 2 to 5 μCi of [^{125}I]staphylococcal A protein (iodinated with Na^{125}I and chloramine T, specific activity about 20 μCi/μg) in rinse buffer. It was rinsed further for 2 h with three changes of rinse buffer and then fluorographed overnight at −20°C with a Dupont Cronex intensifying screen. Lanes a through d, H-RSV(BH) cell extracts; lanes e through g, B77-NRK extracts. Lane a, No addition; lane b, no addition, extract incubated for 2 h at room temperature; lane c, PMSF; lane d, NEM; lane e, no addition; lane f, no addition, extract incubated for 2 h at room temperature; lane g, NEM, PMSF, aprotinin, and EDTA.

The mature virion protein p15, which is generated from the C-terminal moiety of Pr76 in avian cells, is a protease that appears to be involved in the processing of Pr76 (3, 17, 18). The p15 proteolytic activity is inhibited by sulfhydryl reagents like NEM (3, 17). Thus, it might be suggested that the observed instability of Pr76 in extracts of some mammalian cells is a consequence of autoproteolysis. Two arguments can be advanced against this possibility. First, the differences in the stability of Pr76 in the extracts of different cells are difficult to reconcile with this model. Second, we have observed that the avian *gag*-fusion protein in Fujinami sarcoma virus-transformed rat embryo cells also is unstable upon the storage of extracts at −20°C (W. Potts and V. Vogt, unpublished data). As with Pr76, proteolytic breakdown can be inhibited by NEM. This protein, which comprises approximately the N-terminal half of Pr76 fused to a cellular protein that is responsible for transformation, does not contain the p15 moiety. Thus, it seems likely that a cellular protease is responsible for Pr76 degradation in vitro. We infer that this enzyme bears an active sulfhydryl group and is present at elevated levels in rat kidney cells. Further, it is presumably sequestered from Pr76 in vivo, since no partial digestion fragments were detectable by blottings (Fig. 1 and 2) and since, at least in hamster cells, Pr76 is stable in pulse-chase experiments (5).

The apparent inability of mammalian cells to process Pr76 correctly, which has also been noted cursorily by others (1, 9), seems likely to be related to the lack of assembly of avian virus particles in these cells. The processing defect could be either the cause or the effect of this assembly block. According to the latter model, cleavage of Pr76 can occur only in the final stages of, or subsequent to, the budding of virus particles from the plasma membrane. Thus, for example, if Pr76 were unable to interact with the

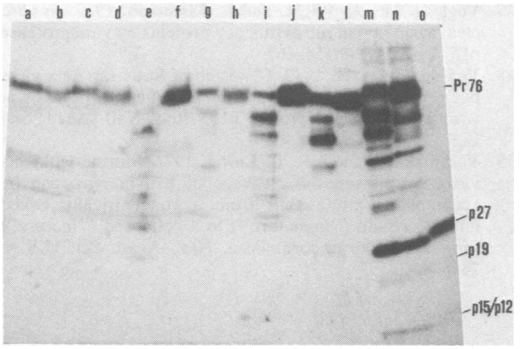

FIG. 2. Stability of Pr76 in different mammalian cells. Crude cytoplasmic fractions from RSV-transformed mammalian cells grown in fetal calf serum [H-RSV(BH) and HF-RSV] or calf serum (other cells) were prepared as described in the legend to Fig. 1. The freshly prepared extracts were divided into portions of 40 μl and frozen at −20°C. After several days, the samples from KC, SR-NRK, SR-BALB, and HF-RSV were lyophilized and then redissolved in 40 μl of 5% SDS–2% mercaptoethanol sample buffer. The samples of SR-Q, H-RSV(BH), and B77-NRK were thawed in parallel, and then portions of 7, 5, or 20 μl, respectively, were added to 20 μl of 10% SDS–4% mercaptoethanol sample buffer. Gel electrophoresis, blotting, and staining with anti-*gag* serum and [^{125}I]staphylococcal A protein were performed as described in the legend to Fig. 1. In lanes a, c, e, g, i, k, and m, the lysis buffer contained no additions. In lanes b, d, f, h, j, l, and n, it contained NEM, aprotinin, EDTA, and PMSF as described in the legend to Fig. 1. Lanes a and b, HF-RSV (Rhesus); lanes c and d, SR-BALB (mouse); lanes e and f, SR-NRK (clone A_4B_5, rat); lanes g and h, KC (human); lanes i and j, B77-NRK (rat); lanes k and l, H-RSV(BH) (hamster); lanes m and n, SR-Q (a line of RSV-transformed quail cells); lane o, 0.04 μg of avian myeloblastosis virus as marker. The SR-NRK cells were obtained from L. Rohrschneider, and the SR-Q cells were obtained from K. Steimer; the origin of the other cell lines is given in Table 1, footnote *b*.

proper plasma membrane components in mammalian cells, then it might not achieve the configuration needed for budding and, thus, would not be subject to cleavage. The fact that amphibian oocytes injected with RSV RNA synthesize and also cleave Pr76 into mature *gag* proteins (8) would argue against this model, if our presumption that virus particles are not formed in oocytes could be verified.

In an alternative model, the cleavage defect itself leads to the failure of virus particles to bud from the membrane. Murine leukemia viruses (MuLV) are able to assemble without the cleavage of Pr65, the MuLV *gag* precursor analogous to Pr76, resulting in virus particles that have a so-called immature or A-type morphology by electron microscopy (19). However, these observations on MuLV do not contradict the assumptions of this model, since avian sarcoma or leukemia viruses have never been observed either to contain uncleaved Pr76 or to form A-type particles. In a particular formulation of this model, a cellular protease that is present in avian, but not in mammalian, cells initiates the budding process by cleaving p15 from Pr76, thereby activating the p15 proteolytic activity. Preliminary experiments have shown that avian cell extracts contain an enzymatic activity capable of generating a polypeptide the size of p15 from the C-terminal end of Pr76 (V. Vogt and A. Wight, unpublished data; R. Eisenman, personal communication). However, no direct evidence has yet been presented to support or contradict either of the two models.

We acknowledge the helpful discussions with D. Boettiger, R. B. Pepinsky, and R. Eisenman, and we thank the several people who supplied cell lines.

This work was supported by grant CA 20081 awarded by the U. S. Public Health Service.

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